

### **REMARKS**

In this amendment, Applicants have currently cancelled claims 37, 39, 41, 42 and 43, and have currently amended claim 40, all for the sole purpose of expediting prosecution. Applicants preserve the right to prosecute all cancelled claims and deleted subject matter in continuing patent applications.

#### **Objection to the Title of the Invention**

The Examiner alleged that the title is not descriptive of the claims. Applicants respectfully disagree. All of the compounds disclosed and claimed in the instant application are P70S6 kinase modulators, as indicated in the title. In addition, the instant application also discloses methods of using the claimed compounds, as indicated in the title. Paragraph [0199] described what IC<sub>50</sub> values are (i.e., IC<sub>50</sub> is the inhibitor concentration at 50% enzyme inhibition). The compounds in the application have been tested for the inhibition of P70S6 kinase as measured by the P70S6 kinase IC<sub>50</sub> value assay described in paragraph [0200]. The P70S6 kinase IC<sub>50</sub> values for the claimed compounds are listed in Table 2. Since the definition of modulation includes inhibition (see paragraph [0011] of the instant application), the title is descriptive of what the claimed compounds are. Accordingly, Applicants respectfully request withdrawal of this objection.

#### **Objection to the Abstract of the Disclosure**

The Examiner has requested that a structural formula be included in the Abstract of the Disclosure. In response, Applicants have amended the Abstract of the Disclosure to disclose the generic compound of the first claim within the amended set of claims. Accordingly, Applicants respectfully request reconsideration and withdrawal of this objection.

#### **Rejections under 35 U.S.C. § 112**

(1) The Office rejected claims 37 and 39-43 under 35 U.S.C. § 112, first paragraph, alleging that the specification does not reasonably provide enablement. Applicants respectfully traverse these rejections.

### **The Legal Standard for Enablement**

The test for enablement is whether one reasonably skilled in the art could make or use the invention, without undue experimentation, from the disclosure in the patent specification coupled with information known in the art at the time the patent application was filed. *U.S. v Telectronics Inc.*, 857 F.2d 778, 8 U.S.P.q.2d 1217 (Fed. Cir. 1988). In fact, well known subject matter is preferably omitted. *See Hybritech Inc., v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384 (Fed. Cir. 1986) (“a patent need not teach, and preferably omits, what is well known in the art”). The determination of enablement is based on whether a person skilled in the pertinent art could make and use the invention without undue experimentation. *See Northern Telecom, Inc. v Datapoint Corp.*, 908 F.2d 931 (Fed. Cir. 1990). The applicant(s) for a patent application need not set forth every minute detail regarding the invention. *Phillips Petroleum Co. v. United States Steel Corp.*, 673 F. Supp. 1278, 1291 (D. Del. 1991).

Undue experimentation is experimentation that would require a level of ingenuity beyond from what would be expected from one of ordinary skill in the art. *Fields v. Conover*, 170 U.S.P.Q. 276, 279 (C.C.P.A. 1971). The fact that experimentation may be complex does not make it undue, so long as the art typically engages in such experimentation. *In re Certain Limited-Charge Cell Culture Microcarriers*, 221 USPQ 1165, 1174. The factors that may be considered in making a determination of whether an amount of experimentation is undue are listed in *In re Wands*, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988). Among these factors are: the amount of effort involved, the breadth of the claims, the level of predictability in the art, the guidance provided by the specification, the presence of working examples, the amount of pertinent literature, and the level of skill in the art. The test for undue experimentation is not merely quantitative, since a considerable amount of experimentation is permissible, so long as it is merely routine. *Id.*

Further, while the predictability of the art can be considered in determining whether an amount of experimentation is undue, mere unpredictability of the result of an experiment is not a consideration. Indeed, the Court of Custom and Patent Appeals in *In re Angstadt*, has specifically cautioned that the unpredictability of the result of an

experiment is not a basis to conclude that the amount of experimentation is undue. In particular, in an unpredictable art, it is not necessary for an inventor to disclose a test with every species covered by a claim, as it would force an inventor seeking patent protection to carry out a prohibitive number of experiments, which could discourage inventors from filing patent applications in technical areas that may be unpredictable. In re Angstadt, 190 U.S.P.Q. 214(C.C.P.A. 1976).

Applicants have cancelled claims 37, 39, 41, 42 and 43, thereby rendering the rejection to these claims moot. In addition, Applicants have amended claim 40 to include specific indications for which there is an association between the activity of p70S6K and these diseases, which are cancer, rheumatoid arthritis, graft-host diseases, multiple sclerosis, psoriasis, arteriosclerosis, myocardioinfarction, ischemia, stroke, diabetes, obesity, hypercholesterolemia, interbowel diseases, osteoarthritis, macular degeneration and diabetic retinopathy.

For example, paragraph [0012] provides an example of the association between p70S6K activity and cancer which was reported by Peralba et al (2003) Clinical Cancer Research 9, 2887. A role for p70S6K in tumor cell proliferation and protection of cells from apoptosis is also supported based on its participation in growth factor receptor signal transduction, overexpression and activation in tumor tissues [Pene et al (2002) Oncogene 21, 6587; Miyakawa et al (2003) Endocrin J. 50, 77, 83; Le et al (2003) Oncogene 22, 484]. A role for p70S6K in abnormal cell growth and proliferation is supported based on these findings.

Also, p70S6K was found to be implicated in metabolic diseases and disorders. It was reported that the absence of P70S6K protects against age- and diet-induced obesity while enhancing insulin sensitivity [Um et al (2004) Nature 431, 200-205 and Pende et al (2000) Nature 408, 994-997]. A role for p70S6K in metabolic diseases and disorders such as obesity, diabetes, metabolic syndrome, insulin resistance, hyperglycemia, hyperaminoacidemia, and hyperlipidemia is supported based upon the findings. In addition, it was reported that p70S6K is implicated in Angiotensin II induced protein synthesis in rat aortic vascular smooth muscle cell and cardiac myocytes. (See *BMC Cardiovascular Disorders* 2004, 4:6, a copy of which is enclosed).

The specification fully enables Claim 40 as amended. There are many specific compounds in claim 34 that support the generic scope of claim 13, and these compounds were tested to be active in modulating (inhibiting) p70S6K in terms of their p70S6K IC<sub>50</sub> values (see Table 2).

Applicants assert that the breadth of amended claim 40 is very specific towards a method of treating the diseases listed in amended claim 40. The predictability is satisfied by the numerous compounds in the claims that can inhibit p70S6K, wherein compounds that can inhibit p70S6K are associated with being able to treat the diseases or disorders listed in amended claim 40. Each compound in claim 34 is a working example of which compounds can be used to treat the diseases or disorders listed in amended claim 40.

For all the reasons stated above, Claim 40 satisfies the enablement requirement of 35 U.S.C. § 112, first paragraph. Applicants respectfully request reconsideration and withdrawal of this rejection.

Applicants respectfully request that the present amendments and remarks be entered and made of record in the instant application. Withdrawal of the Examiner's rejections and allowance of this patent application is respectfully requested. The Examiner is invited to telephone the undersigned to discuss any remaining issues in connection with this patent application.

Respectfully submitted,

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Research article

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## Signaling of angiotensin II-induced vascular protein synthesis in conduit and resistance arteries *in vivo*

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### Abstract

**Background:** From *in vitro* studies, it has become clear that several signaling cascades are involved in angiotensin II-induced cellular hypertrophy. The aim of the present study was to determine some of the signaling pathways mediating angiotensin II (Ang II)-induced protein synthesis *in vivo* in large and small arteries.

**Methods:** Newly synthesized proteins were labeled during 4 hours with tritiated leucine in conscious control animals, or animals infused for 24 hours with angiotensin II (400 ng/kg/min). Hemodynamic parameters were measured simultaneously. Pharmacological agents affecting signaling cascades were injected 5 hours before the end of Ang II infusion.

**Results:** Angiotensin II nearly doubled the protein synthesis rate in the aorta and small mesenteric arteries, without affecting arterial pressure. The AT<sub>1</sub> receptor antagonist Irbesartan antagonized the actions of Ang II. The Ang II-induced protein synthesis was associated with increased extracellular signal-regulated kinases (ERK)1/2 phosphorylation in aortic, but not in mesenteric vessels. Systemic administration of PD98059, an inhibitor of the ERK-1/2 pathway, produced a significant reduction of protein synthesis rate in the aorta, and only a modest decrease in mesenteric arteries. Rapamycin, which influences protein synthesis by alternative signaling, had a significant effect in both vessel types. Rapamycin and PD98059 did not alter basal protein synthesis and had minimal effects on arterial pressure.

**Conclusion:** ERK1/2 and rapamycin-sensitive pathways are involved in pressure-independent angiotensin II-induced vascular protein synthesis *in vivo*. However, their relative contribution may vary depending on the nature of the artery under investigation.

## Background

Angiotensin II (Ang II) has an important role in the physiological and pathophysiological regulation of the arterial wall. Indeed, in addition to being a vasoactive peptide, this multifunctional hormone stimulates hypertrophy of isolated vascular smooth muscle cells (VSMC), as a result of enhanced protein synthesis [1]. Chronic administration of Ang II has been shown to promote significant changes in vascular structure, leading to pressure-independent hypertrophic remodeling of small arteries [2-4]. In large arteries, exogenous Ang II induces hypertrophy followed by increased DNA synthesis [5,6].

Binding of Ang II to the Ang II subtype 1 ( $AT_1$ )-receptor triggers a complex series of intracellular signaling events activating protein kinase cascades acting synergistically to increase the rate of global protein synthesis [7,8]. *In vitro* studies identified at least two signaling pathways directly linked to protein synthesis [9-11]. Activation of members of the mitogen-activated protein kinase (MAPK) family, of which extracellular signal-regulated kinase-1 (ERK-1 or  $p44^{mapk}$ ) and ERK-2 ( $p42^{mapk}$ ) represent one of these pathways [10,12]. Their threonine/tyrosine phosphorylation and activation by MEK can be pharmacologically inhibited by the synthetic compound PD98059 [9,13,14]. Activation of the  $AT_1$  receptor also stimulates the phosphorylation and enzymatic activity of the 70-kD S6 kinase ( $p70^{S6k}$ ) in VSMC [10], which is the major physiologic kinase for ribosomal protein S6, a component of the 40S ribosomal subunit [15].  $p70^{S6k}$  is implicated in Ang II-induced protein synthesis in rat aortic VSMC and cardiac myocytes [10,11]. Indeed, *in vitro* studies have shown that rapamycin, an immunosuppressive agent, abolishes activation (phosphorylation) of  $p70^{S6k}$ , and consequently of protein synthesis [10,11].

Thus, although *in vitro* studies clearly indicate that Ang II activates cascades involving ERK-1/2 and  $p70^{S6k}$ , which both contribute to enhance protein synthesis, little is known about the *in vivo* contribution of these signaling pathways to the vascular effect of Ang II. Furthermore, a different contribution of signaling cascades in arteries with different physiological function is a plausible hypothesis that deserves investigation. These issues represent the aim of the present study and to address them, we used a model that allows the measurement of vascular protein synthesis *in vivo*. We then compared the efficacy of PD98059 and rapamycin to modulate protein synthesis in conduit (aorta) and resistance (small arteries from the mesenteric circulation) vessels.

## Methods

### Animals and treatments

Male Sprague-Dawley rats weighing 300–325 g (obtained from Charles River Laboratories, Que., Canada) were

anesthetized with pentobarbital sodium (65 mg/kg, i.p.) for insertion of a polyethylene catheter (PE10 segment welded to a PE50) into the femoral artery and vein. In some animals, an osmotic pump (model 1003D Alzet®) was simultaneously implanted subcutaneously in the subcostal region, releasing a constant dose of 400 ng/kg/min of Angiotensin II. Rats were then free to move and had access to food and water, with a tethering system protecting the catheters [16].

Twenty two hours after surgery, a saline solution containing L-(3,4- $^3H$ ) leucine was infused i.v. for 4 hours at a rate of 12  $\mu$ Ci/hour. Other pharmaceutical agents were administered by i.v. bolus injection following 21 hours of Angiotensin II infusion (one hour prior to [ $^3H$ ]-leucine infusion). Ang II-treated rats received the synthetic compound PD98059 at doses of 1 mg/kg ( $n = 6$ ), 5 mg/kg ( $n = 7$ ) and 10 mg/kg ( $n = 6$ ). A group of control rats received 10 mg/kg PD98059 ( $n = 4$ ). In a second set of experiments, rapamycin was injected at doses of 0.1 mg/kg ( $n = 8$ ), 0.5 mg/kg ( $n = 6$ ) and 1 mg/kg ( $n = 3$ ) in Ang II-treated rats. Six control rats received 0.5 mg/kg rapamycin. In a third series, Ang II-treated rats received irbesartan, a selective  $AT_1$  receptor blocker, at doses of 10 mg/kg ( $n = 9$ ), 30 mg/kg ( $n = 5$ ) and 40 mg/kg ( $n = 5$ ), following the same experimental protocol. Additional rats were treated with irbesartan according to a different treatment scheme: Irbesartan was administered subcutaneously at the time of surgery and 12 hours later (10 mg/kg at each occasion) in Ang II-treated ( $n = 8$ ) or in control rats ( $n = 6$ ). We used two sets of control and Ang II-treated rats to confirm the reproducibility of the method. The first set ( $n = 10$  and 10, respectively) was studied simultaneously with PD98059 and irbesartan groups. The second set ( $n = 7$  and 9, respectively) was studied in parallel with the rapamycin experiments. Additional control ( $n = 3$ ) and Ang II-treated ( $n = 3$ ) rats were sacrificed 21 hours after the beginning of Ang II administration, to determine ERK-1/2 phosphorylation at the time when PD98059 was normally injected. Finally, in 3 control and 3 rats treated for 5 hours with PD98059, we confirmed the *in vivo* effectiveness of PD98059 to reduce basal ERK-1/2 phosphorylation (data not shown).

Mean arterial pressure (MAP) was continuously measured intra-arterially in freely moving rats 15 minutes before and averaged for the 5 hours following drug administration. The animals were then anesthetized (pentobarbital 35 mg/kg i.v.) and exsanguinated. The thoracic aorta and the mesenteric vascular bed were collected and immediately transferred in a modified cold Krebs-Ringer bicarbonate solution (composition in mmol/L: NaCl 118.6; KCl 4.8;  $CaCl_2$  2.5;  $MgSO_4$  1.2;  $KH_2PO_4$  1.2;  $NaHCO_3$  25.1;  $Na^+$ ,  $Ca^{2+}$ -EDTA 0.026; glucose 10.1). The aorta and small ramifications of the superior mesenteric artery (first order and smaller) were freed from surrounding tissue and frozen in

liquid nitrogen. The Animal Care and Use Committee at the Université de Montréal approved all the study protocols.

#### **Protein synthesis measurement**

In order to measure leucine incorporation in small and conduit arteries, we used a method derived from that of McNulty et al. [17]. Previous publications present additional experiments that validate the method in different conditions, including Ang II infusion [18,19]. Briefly, tissues were pulverized with dry ice and liquid nitrogen. Five volumes 10% trichloroacetic acid (TCA) were added and the samples were left overnight at 4°C. Tissues were then rinsed once in the same amount of 10% TCA and twice in water to wash non-incorporated leucine. The pellet was solubilized in potassium hydroxide (KOH 1 M) and radioactivity was measured. Results obtained are in cpm/mg of tissue. The second portion of pulverized tissue was also left overnight in 10% TCA, and then solubilized in sodium hydroxide (NaOH 1 M) for measurement of protein content by the method described by Lowry [20]. Results obtained were in mg of proteins/mg of tissue. The final data is expressed as CPM/mg protein and represents the rate of protein synthesis over a 4-hour period.

#### **Determination of ERK-1/2 phosphorylation**

To confirm that Ang II stimulates the ERK-1/2 pathway and that PD98059 is effective *in vivo*, ERK-1/2 phosphorylation was determined in vascular tissues by western blot, using a phosphospecific antibody, as previously described [21].

#### **Drugs and statistical analysis**

All drugs were purchased from Calbiochem. Irbesartan was a kind gift from Bristol-Myers Squibb. PD 98059 was suspended in a 1% polymeric solution (Pluronic F68) and sonicated (ultrasound) prior to administration. We previously confirmed that the vehicle had no effect on protein synthesis. Rapamycin was suspended in 0.2% carboxymethylcellulose (CMC) by sonication, aliquoted and frozen. Prior to its administration, rapamycin was further diluted in CMC.

Data are presented as mean  $\pm$  s.e.m. Statistical analysis was done by ANOVA followed by Bonferroni's correction for multiple comparisons. A priori comparisons were: Ang II and drugs alone vs control, and drugs + Ang II vs Ang II alone.  $P < 0.05$  was considered significant.

### **Results**

#### **ERK-1/2 pathway**

Angiotensin II increased the rate of protein synthesis by 72% in the aorta and by 80% in mesenteric arteries (Figure 1). At the dose used, Ang II administration did not elevate arterial pressure as compared to control rats (Table

1). PD98059, a selective MEK inhibitor, had no significant effect on aortic and mesenteric protein synthesis when administered alone at 5 mg/kg (Figure 1). Given at doses of 1 and 5 mg/kg, PD98059 had no significant effect on Ang II-induced protein synthesis in the aorta and mesenteric bed. At 10 mg/kg, the MEK inhibitor did not produce a further reduction in protein synthesis in small arteries, but totally blocked aortic Ang II-induced protein synthesis (107% reduction). The effect obtained at 10 mg/kg but not at smaller doses confirms that the vehicle, the volume of which was the same for each injection, does not alter protein synthesis. Antibodies directed against the active (phosphorylated) form of ERK-1/2 confirmed that their aortic activity doubled following Ang II administration (Figure 2). In contrast, however, ERK-1/2 activity was not enhanced in mesenteric arteries. The acute administration of PD98059 did not influence mean arterial pressure averaged over the five hours following its administration (Table 1).

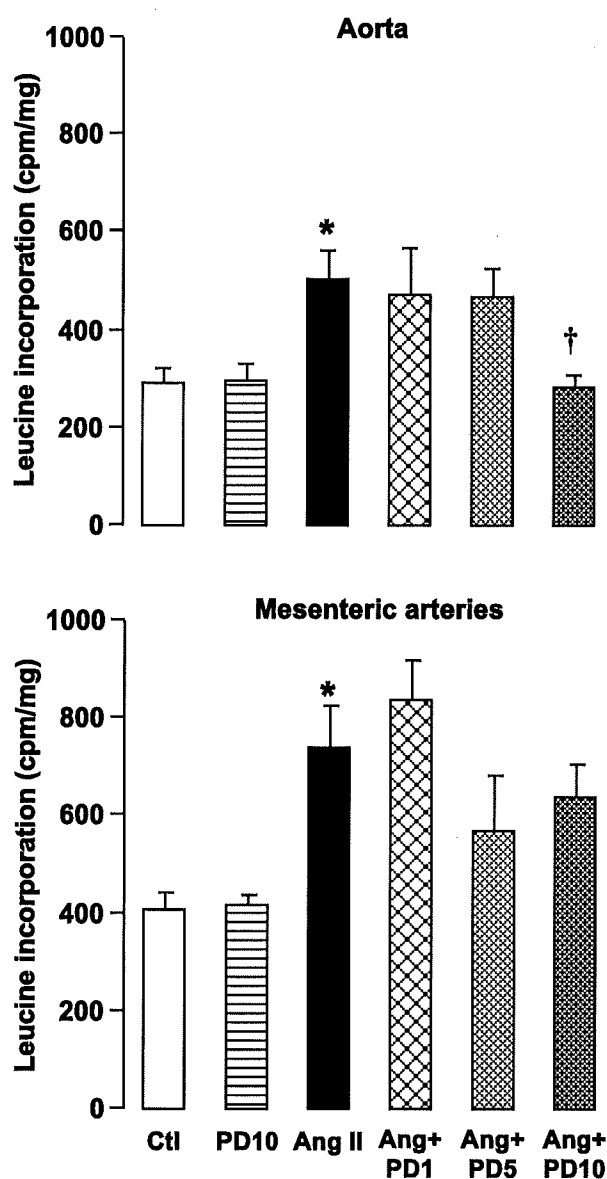
#### **Rapamycin-sensitive pathway**

In this second series, Ang II enhanced aortic and mesenteric protein synthesis by 91 and 105%, respectively (Figure 3), confirming the reproducibility of our method. When administered alone as an i.v. bolus, 0.5 mg/kg rapamycin had no effect on protein synthesis in the mesenteric bed, nor in the aorta (Figure 3). When protein synthesis was first stimulated by Ang II, rapamycin produced a significant reduction in mesenteric arteries, reaching a maximum of 84% at 0.5 mg/kg. As with PD98059, the effect in the aorta improved further at the highest dose, reaching 76% reduction at 1 mg/kg. The highest dose of rapamycin increased mean arterial pressure by 10 mmHg averaged over the course of its administration (Table 1).

#### **AT-1 Receptor antagonism**

Using the same protocol as in the previous experiments, we administered irbesartan, a specific and selective AT<sub>1</sub> receptor blocker, one hour prior to leucine infusion. In both aorta and small mesenteric arteries, irbesartan at 10, 30 and 40 mg/kg had no significant effect on Ang II-induced increase in protein synthesis (data not shown). However, when administered at the beginning and 12 hours after the start of Ang II infusion ( $2 \times 10$  mg/kg), irbesartan totally abolished the trophic effect of Ang II in both vascular beds (Figure 4). In addition, irbesartan administered alone had a tendency to reduce protein synthesis in the aorta. This tendency reached statistical significance in the mesenteric arteries. The blood pressure lowering effect of irbesartan was similar in both treatment regimens (Table 1).





**Figure 1**  
Effect of PD98059 (PD at 1, 5 and 10 mg/kg) on aortic and mesenteric leucine incorporation (expressed in cpm/mg protein). The effect of the drug was tested in basal conditions and after Angiotensin II (Ang)-induced protein synthesis. \*  $P < 0.05$  vs Control (Ctl); †  $P < 0.05$  vs Ang II alone (ANOVA + Bonferroni).

## Discussion

Ang II is a potent trophic factor in several cell types, including VSMC [1,10]. Chronic *in vivo* administration of the peptide produces a pressure-independent hypertrophy

of the vascular wall [2-4,22]. Both hypertrophy and DNA synthesis have been reported in large arteries, although protein synthesis seems to precede DNA replication [5,6]. In agreement with *ex vivo* protein synthesis measurement in tissues extracted from rats treated with Ang II [23], our study demonstrates increased protein synthesis *in vivo* following Ang II administration in conduit and resistance arteries. The enhanced protein synthesis occurred without any significant change in arterial pressure, supporting the concept that Ang II exerts a vascular trophic action independently from pressure changes [2,4,22].

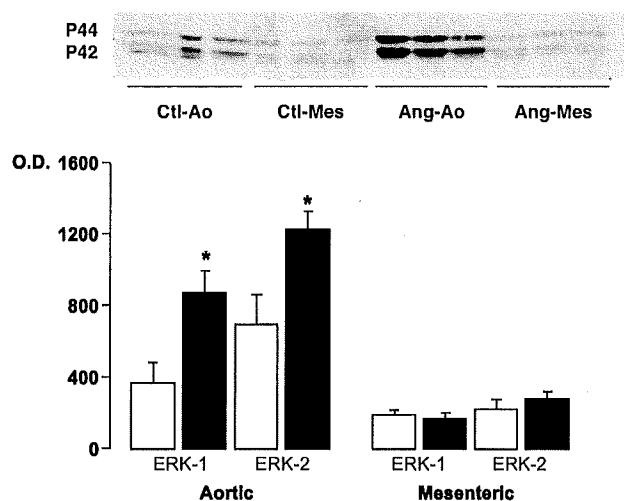
Angiotensin II initiates complex cellular signaling events, and it appeared imperative to determine which pathways are operative *in vivo*, while also examining for potential vascular heterogeneity. The ERK1/2 pathway has been implicated in Ang II-induced protein synthesis [9], and the development of PD98059, which inhibits the phosphorylation and activation of ERK by MEK [9,13,14], has been a key element to reveal these findings. We have previously reported that the highest dose of PD98059 inhibits basal and stimulated ERK 1/2 phosphorylation *in vivo* [19]. Our results with the antagonist strongly suggest that this pathway is an important pressure-independent component of Ang II-induced elevation of aortic protein synthesis *in vivo*. The significant increase in ERK 1/2 phosphorylation following Ang II administration is also in line with such an interpretation. However, in small arteries PD98059 had only a modest effect on Ang II-induced protein synthesis. This correlates well with the apparent inability of Ang II to stimulate ERK-1/2 activity in these vessels, as demonstrated in the present study. Our results contrast those published recently, showing that Ang II stimulates ERK 1/2 activity in small isolated mesenteric arteries [24]. However, the time course was very different (see discussion below).

Rapamycin is another compound that inhibits VSMC protein synthesis induced by several growth factors, including Ang II [10,15]. *In vivo* administration of rapamycin several hours after Ang II showed a marked inhibition of Ang II-induced protein synthesis in mesenteric arteries and in the aorta. This effect occurred without a reduction of arterial pressure, such as seen with irbesartan. In fact, with the highest dose of rapamycin arterial pressure was even elevated, an effect which could explain the loss of a significant reduction of protein synthesis in small arteries at this dose. The low number of animals may also contribute to the statistical outcome. Thus, the rapamycin-sensitive signaling cascade is a second pathway mediating protein synthesis in large arteries *in vivo*, but may be predominantly responsible for the Ang II response in small arteries. One likely explanation for the regional heterogeneity could be related to differences in the machinery regulating protein synthesis. ERK 1/2 appear to converge towards the

**Table 1: Mean arterial pressure before and after drug administration.**

| Treatment                 | MAP: pre-drug (mm Hg) <sup>†</sup> | MAP: post-drug (mm Hg) |
|---------------------------|------------------------------------|------------------------|
| Control                   |                                    | 103 ± 5                |
| Ang II                    |                                    | 105 ± 7                |
| Ang II + PD 1 mg/kg       | 122 ± 6                            | 117 ± 10               |
| Ang II + PD 5 mg/kg       | 119 ± 4                            | 111 ± 5                |
| Ang II + PD 10 mg/kg      | 115 ± 4                            | 113 ± 2                |
| Irb 2 × 10 mg/kg          |                                    | 75 ± 1                 |
| Ang II + Irb 2 × 10 mg/kg |                                    | 84 ± 2                 |
| Control                   |                                    | 112 ± 3                |
| R 0.5 mg/kg               | 98 ± 10                            | 95 ± 10                |
| Ang II                    |                                    | 114 ± 7                |
| Ang II + R 0.1 mg/kg      | 101 ± 9                            | 93 ± 9                 |
| Ang II + R 0.5 mg/kg      | 109 ± 4                            | 109 ± 2                |
| Ang II + R 1.0 mg/kg      | 111 ± 5                            | 123 ± 1 *              |

Data are presented as mean ± sem \*  $P < 0.05$  vs Pre-drug (paired t-test). † For control and Angiotensin II-treated animals that did not receive further acute treatments, only one pressure is presented. MAP Pre-drug: mean arterial pressure before acute drug administration; MAP post-drug: MAP averaged during the 5 hours following acute drug administration; PD: PD98059; R: Rapamycin; Irb: Irbesartan.

**Figure 2**

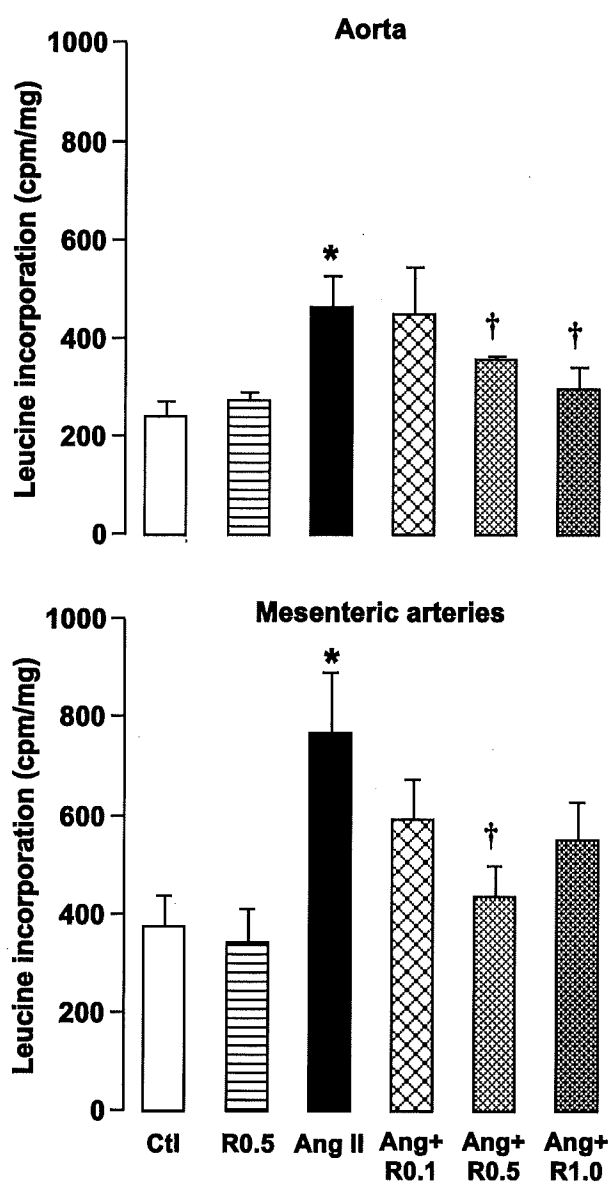
Western blot analysis with a phosphospecific antibody of protein samples prepared from three control aortas (Ctl-Ao, lanes 1–3) and mesenteric arteries (Ctl-Mes, lanes 4–6), and three angiotensin II-treated aortas (Ang-Ao, lanes 7–9) and mesenteric arteries (Ang-Mes, lanes 10–12). The bar chart represents the mean optical density (O.D.) of the three experiments for both ERK-1 (p44<sup>mapk</sup>) and ERK-2 (p42<sup>mapk</sup>) in control (open bars) and Ang II-treated animals (filled bars). \*  $P < 0.05$  vs Ctl; (unpaired t-test).

activation of the mRNA 5' cap-binding protein eIF4E, which seems to be the rate-limiting step in cap-dependent mRNA translation [8,25]. In contrast, rapamycin inhibits

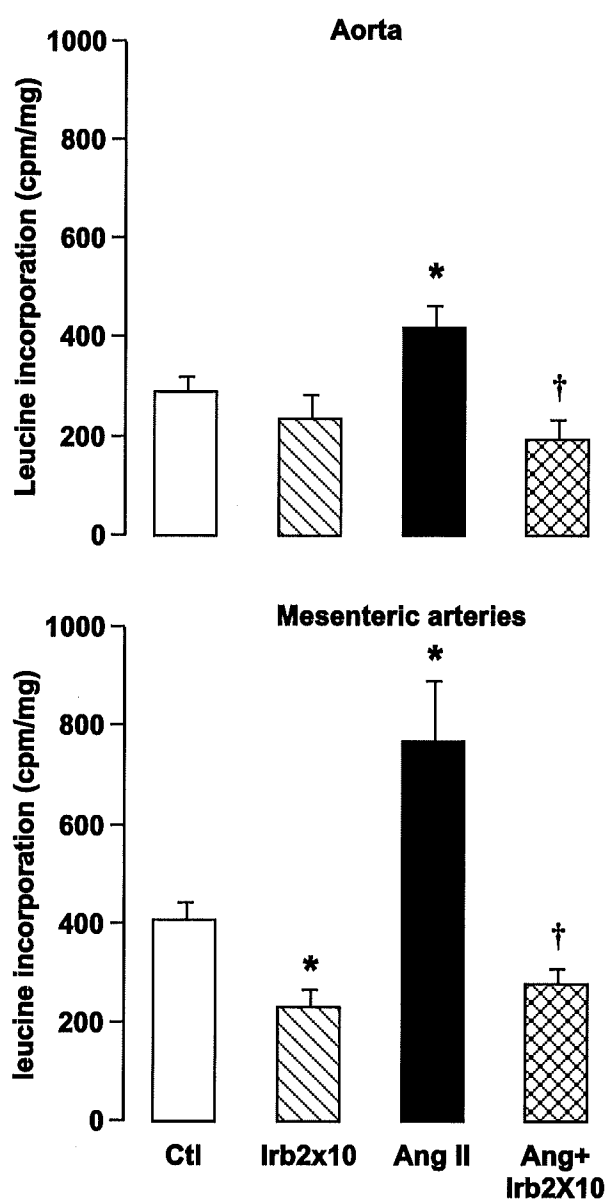
FRAP/mTOR, which lies in the cascade relaying phosphoinositide 3-OH kinase (PI 3-K) to the phosphorylation and inactivation eIF4E-binding protein (4E-BP1) [26] and to the activation of the p70<sup>s6k</sup>, which also regulates the translation of a subset of mRNAs [27]. Thus, it is possible that VSMC in different types of arteries, with distinct physiological roles, may actually regulate their protein synthesis by alternative mechanisms. The present study only suggests this heterogeneity and a more appropriate study will be designed to address this hypothesis, which could be of future therapeutic importance. Indeed, different relative cellular composition of the vessel wall could also explain our observation.

In our *in vivo* conditions, ERK1/2 and rapamycin-sensitive pathways seemed to overlap to a great extent in the aorta. Considering the magnitude of inhibition by both drugs alone, combination of PD98059 and rapamycin would not provide additional information on the interdependence of the signaling events *in vivo*. An overlap of smaller magnitude has also been reported in cell culture systems, although the pathways were shown to be independent [9], since rapamycin did not modify ERK1/2 activity [9]. However, a more recent study suggests that the ERK-1/2 pathway could actually enhance p70<sup>s6k</sup> phosphorylation following Ang II administration, through EGF-receptor transactivation [28]. It is not known if this translates into enhanced protein synthesis, but our results seem consistent with an interdependence of the two pathways in the control of aortic protein synthesis.

The role of AT<sub>1</sub>-receptors mediating Ang II-induced protein synthesis was confirmed with irbesartan, a potent AT<sub>1</sub>-receptor antagonist [29,30]. In addition, the results



**Figure 3**  
Effect of Rapamycin (R at 0.1, 0.5 and 1.0 mg/kg) on aortic and mesenteric leucine incorporation (expressed in cpm/mg protein). The effect of the drug was tested in basal conditions and after angiotensin II (Ang)-induced protein synthesis. \*  $P < 0.05$  vs Ctl; †  $P < 0.05$  vs Ang II alone (ANOVA + Bonferroni).



**Figure 4**  
Effect of Irbesartan (Irb, 10 mg/kg s.c. twice at 12 hours interval) on basal and angiotensin II (Ang II)-stimulated aortic and mesenteric leucine incorporation (expressed in cpm/mg protein). \*  $P < 0.05$  vs Ctl; †  $P < 0.05$  vs Ang II alone (ANOVA + Bonferroni).

suggest that endogenous Ang II exerts a tonic effect on protein synthesis, as basal protein synthesis was inhibited by irbesartan. However, this could also be due to the hypotensive effect of irbesartan, as a change in hemody-

namic conditions is likely to influence the vessel wall. Although our initial studies (5 hour administration) did not show any influence of pressure reduction on vascular protein synthesis *per se*, the longer period of hypotension observed in the second series of experiments (24 hours of

Irbesartan) could still explain the reduction of protein synthesis below physiological levels. Alternatively, unopposed AT<sub>2</sub> receptors may reduce proliferation during AT<sub>1</sub>-receptor blockade, as recently suggested by the reduced efficacy of an AT<sub>1</sub> receptor antagonist to prevent hypertrophy of coronary arteries in AT<sub>2</sub> receptor null mice [31]. Interestingly, blockade of the ERK-1/2 or the rapamycin-sensitive pathways did not influence basal protein synthesis in large and small arteries, suggesting that these two pathways, which respond to trophic stimuli, may not be key elements in protein renewal under physiological conditions, and therefore represent potential therapeutic targets.

Because of the complex cellular microenvironment produced by local and circulating factors, the direct relationship between an agonist and effector pathways cannot be resolved when working *in vivo*. Furthermore, signaling cascades and their kinetic of activation cannot be studied as thoroughly as when using cell culture systems. Consequently, the aim of the present study was not to characterize the signaling events up- or downstream of the drugs' site of action, but to identify the pathways operating *in vivo* in arteries with distinct physiological functions. Our interpretation of the data is based on the current understanding of the mechanism of action of the pharmacological agents that were used either to antagonize AT<sub>1</sub>-receptors or signaling events. In that respect, it was recently shown that PD98059 can also affect ERK5 [32], the contribution of which is not known in our study.

It must also be underscored that the pharmacokinetic profile of drugs affecting signaling cascades is seldom known and the *in vivo* protocols have to be devised with short duration between drug administration and end-point measurement. This explains the rather late administration of PD98059 and rapamycin with respect to Ang II infusion. In fact, the effect of PD98059 on protein synthesis was rather surprising, considering that *in vitro* Ang II-induced activation of ERK-1/2 is transient in nature and fades after 60 minutes [12]. However, it is consistent with a report demonstrating sustained ERK-1/2 activity elevation in the aorta of Dahl and stroke-prone spontaneously hypertensive rats [33]. Furthermore, we confirmed that ERK-1/2 phosphorylation was elevated in the aorta after 21 hours of Ang II administration, corresponding to the injection time of PD98059 or rapamycin. It is thus likely that with constant infusion of Ang II *in vivo* ERK-1/2 activation follows a different kinetic than in synchronized cells in culture receiving a single dose of Ang II. In small arteries, we could not observe a sustained elevation of ERK-1/2 phosphorylation, explaining, at least in part, the lack of efficacy of PD98059 to reduce protein synthesis. However, a recent report demonstrated that Ang II application to isolated and pressurized small arteries did

enhance ERK-1/2 activity after 5 minutes [24]. In that study, PD98059 reduced Ang II-induced vasoconstriction, suggesting that ERK-1/2 may mediate different cellular effects, and hence follow different activation kinetics in large and small arteries. To further support this hypothesis, we have recently reported that during acute NOS inhibition, ERK1/2 activation is associated with protein synthesis in large arteries [19], but with vasoconstriction in small resistance arteries [34].

## Conclusion

Our results obtained in *in vivo* conditions demonstrate that Ang II administration at a non-pressor dose enhanced vascular protein synthesis, a necessary first step towards vascular hypertrophy/hyperplasia. In agreement with previous *in vitro* studies, we show that both ERK1/2 and rapamycin-sensitive signaling pathways are involved in Ang II-induced increase in protein synthesis *in vivo*. In contrast to what was suggested from *in vitro* studies, however, late inhibition of the signaling pathways was effective to reduce protein synthesis. In addition, we propose a degree of vascular heterogeneity in the relative implication of ERK1/2 to mediate Ang II-induced protein synthesis. This new information provides further insight on the signaling events mediating hypertrophy of small and large arteries that could be triggered by elevated levels of circulating or local Ang II in pathological conditions.

## Competing interests

None declared.

## Authors' contributions

CD and FMACM performed the treatments for the protein synthesis studies, while DG and HHD performed the western blot analysis. RMT assisted us in the preparation of the manuscript and provided the methodological details of phosphospecific western blots from tissue samples. PM conceived and coordinated the study. All authors read and approved the final version of the manuscript.

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